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THREE METHODS OF USING COTTON BLUE AS A MYCOLOGICAL STAIN.

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THREE METHODS OF USING COTTON BLUE AS A MYCOLOGICAL STAIN.— Cotton blue has frequently been advocated as a stain for fungal hyphae. The stain may be incorporated either in glycerine or lactic acid, and the material to be examined mounted in the coloured liquid or, after staining, transferred to glycerine jelly. When a solution of cotton blue in lactic acid is used as a mounting medium, general morphological structures are very clearly differentiated. In the following article three methods are described which may be employed in preparing permanent preparations of mycelium *in situ* or in the direct examination of fungal hyphae in woody tissues by means of vertical illumination.

(1) Cotton blue is a favourable general cytoplasmic stain for all Phycomycetes, and may well be employed to study the early stages in the formation of the sexual organs of oomycetes growing in culture. The following method has given particularly successful results.

A clean microscope slide is sterilized in a Petri dish which is then filled with about 30 c.c. of clear agar medium. This produces a thin layer over the surface of the slide which should be arranged so that the centre of the slide coincides with the centre of the Petri dish. The medium is now inoculated and incubated. After a suitable period the mycelium in the agar may be stained after fixation with 1 per cent. chromo-acetic acid. The best results have been obtained by placing a Petri dish containing the fixative in the incubator, and when the fluid is warm transferring the thin layer of agar with its supporting slide to the fixative. The slide and agar may be easily dislodged by cutting round the edge of the slide with a scalpel and prising it up from the bottom of the dish. Allow the mycelium to fix for 24 hours at incubator temperature, and then wash thoroughly in gently flowing water. When free of fixative the slide and agar may be placed in 10 per cent. glycerine which is then allowed to concentrate to the strength of Amann's medium. This latter is prepared by dissolving in order,

Carbolic acid crystals	20 grm.
Lactic acid syrup	20 „
Distilled water	20 „
Glycerine (pure)	40 „

Transfer the agar film to a small quantity of this medium in which 0·5 per cent. of cotton blue has been dissolved. Stain for from 6 to 24 hours, according to the

nature of the fungus employed, and then differentiate in several changes of Amann's medium till no further colour is removed from the hyphae. Transfer to 70 per cent. alcohol to wash out the Amann's medium, and bring up through 95 per cent. alcohol to dehydrate in absolute alcohol. If these stages are carefully carried out the agar will still adhere to the slide. Transfer from absolute to 1/3 xylol in 2/3 alcohol, to 2/3 xylol in 1/3 alcohol, and finally clear in pure xylol. When clear, infiltrate the preparation with balsam dissolved in xylol, and allow the excess balsam to drip off the slide. Trim up the edges of the preparation and cover with a large (2 in. × 7/8 in.) cover-glass.

Preparations made in this way give very good results, and if fixation and differentiaion have been thorough, nuclei may be defined by their more intense blue colour. The stain is permanent for at least four years, even if the slides are regularly exposed to light.

The method may be used to investigate early stages in the formation of certain perithecia and pycnidia, but in most cases of this kind the erythrosin staining method described by Gwynne-Vaughan and Barnes has given better results.

(2) The differentiation of fungal hyphae penetrating through woody tissues can be secured by staining them with cotton blue and counterstaining the xylem with safranin. The method described here works equally well with fresh or fixed hyphae and tissue, and may be used to advantage in demonstrating the presence of fungal mycelium in structural timber.

Thin sections of infected tissue are placed in a slightly warm 0·5 per cent. solution of cotton blue in Amann's medium for 5 to 15 minutes. The excess stain is removed by washing in one or two portions of Amann's medium, which is then removed by washing in 70 per cent. alcohol. Transfer the sections from this to a solution of safranin (equal parts of a saturated solution of water-soluble safranin and of a saturated solution of alcohol-soluble safranin). Allow to stain for about 10 minutes. Wash out most of the excess stain in 70 per cent. alcohol and transfer the sections through 95 per cent. alcohol to absolute alcohol. After dehydration clear in xylol and mount in balsam.

The protoplasm of the hyphae stains a brilliant blue colour, contrasting with the red of the xylem. Differentiation is sufficiently marked to yield preparations which can be photographed, and in many cases penetration of the walls of tracheae can easily be observed.

(3) The examination of superficial mycelium by means of low-power objectives using vertical or direct illumination has regularly been employed in mycology, but a similar method of investigating the presence of hyphae upon cut surfaces of infected tissue, and especially timber, has not become a routine practice. This is partly because the normal illumination employed limits the power of objective which can be utilized, and partly because the hyphae are not easily differentiated from the host tissues. By using the Ultropak [1] vertical illuminator, after a pretreatment of the cut surface of the infected tissue with cotton blue, fungal hyphae can be observed under the highest dry objectives, and even under oil immersion objectives.

The method described below has been employed chiefly with woody stems and

[1] Made by Messrs. E. Leitz.

structural timber, storage organs and fleshy fruits, but it may well be extended to herbaceous stems. The infected block of tissue is split through the middle in a radial direction and the cut surface smoothed off with a sharp razor. The prepared surface is then immersed in slightly warm cotton-blue solution in Amann's medium (a 0·5 to 1·0 per cent. solution) for about 10 minutes, and the surplus stain is then washed away in Amann's medium. The block is now placed on a microscope slide and the prepared surface covered with a cover-glass. In the case of woody tissue the infecting hyphae are differentiated by their blue colour against the white of the background, while in storage organs the hyphae are more intensely blue than the general ground tissue.

Under a low power this method can be employed in a qualitative manner to indicate the degree of penetration of fungi inoculated on the surface of wood blocks, storage organs, or fruits. It has been employed to demonstrate the effectiveness of commercial timber preservatives in preventing the attack of wood-destroying fungi. The great asset of the method is the speed with which numerous routine examinations can be carried out.

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